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(71) Applicant: SUNTORY KABUSHIKI KAISHA, also known as SUNTORY LTD.
1-40, Dojimahama 2-chome
Kita-ku Osaka530(JP)

(72) Inventor: Nomura, Midori
20-31, Habikino 4-chome
Habikino Osaka 583(JP)

(72) Inventor: Ohsuye, Kazuhiro
18-7, Inaba-cho
Ibaraki Osaka 567(JP)

(72) Inventor: Tanaka, Shoji
8-46, Minamikaneden-cho 1-chome
Suita Osaka 564(JP)

(74) Representative: Raynor, John et al,
W.H.Beck, Greener & Co 7 Stone Buildings Lincoln's Inn
London WC2A 3SZ(GB)

(54) Improved expression vector and use thereof.

(57) A novel *Escherichia coli* plasmid is described. The plasmid has, between the Shine-Dalgarno sequence and the translation start codon, a base sequence different from the corresponding, originally occurring region in the β-galactosidase operon and contains, upstream therefrom, the region of a hybrid promoter between the *Escherichia coli trp* promoter and the *lac* promoter and, at an entirely independent site, a *lac* promoter repressor gene. Employing an *Escherichia coli* transformant carrying the plasmid, a useful polypeptide such as h-IFN-γ can be produced in high yield.

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Improved expression vector and use thereof

This invention relates to Escherichia coli hybrid
plasmid vectors improved so as to enable high
efficiency production of useful polypeptides and to the
use of said vectors.

5   Heretofore, a number of useful polypeptides have
been produced in microbial or higher animal or plant
cells as hosts with the aid of the so-called
recombinant DNA technology (hereinafter, rDNA
technology), and general techniques therefore can be
10   said to have already been established. However, there
still remains a room for improvement with respect to
hosts and vector systems, among others, for enabling
efficient and economical production in actual
commercialization or application.

15   A variety of factors can be taken into
consideration in relation to efficient production of
useful polypeptides in relation to efficient production
of useful polypeptides using the rDNA technology. As
for expression vectors to be carried by Escherichia
20   coli, for instance, such factors may include the kind
of promoter involved in the promotion of transcription
of the exogeneous gene desired to be expressed, the

base sequence of the so-called Shine-Dalgarno sequence
(hereinafter, SD sequence) involved in complementary
binding of the 3' terminal sequence of 16S ribosome RNA
to mRNA and the distance between said sequence and the
translation start codon (ATG), and the copy number of
plasmid vector. However, for certain polypeptides
desired to be produced in high efficiency, there are no
established rules known yet between these factors and
the productivity of said peptides. For instance, a
certain expression vector capable of causing high
expression of a certain exogeneous gene cannot always
bring about high expression of another exogeneous gene.
In cultivating transformants, an expression vector
capable of inducing efficient introduction and
expression of an exogeneous gene, if available, will
also be important in actual high production of a useful
polypeptide. For example, an expression vector with
which transformant cell growth and plasmid DNA replication
proceed normally without expression of an exogeneous gene
inserted in said vector under certain cultivation
conditions but the transcription of said exogeneous
gene is rapidly accelerated under different cultivation
conditions will be important, because the formation of
a polypeptide, which is often toxic to the host
(transformant), as the product of an exogeneous gene at
the initial stage of cell growth may possibly inhibit

the growth of the host microorganism and as a result cause decrease in productivity.

From the above viewpoint, various modifications of expression vectors have so far been attempted. For instance, there are known a hybrid promoter between the Escherichia coli tryptophan (trp) promoter and the lactose UV5 promoter (tac promoter; cf. H. A. de Boer et al., Proc. Natl. Acad. Sci. USA, 50, 21, 1983 and Japanese Patent Application Kokai Tokkyo Koho No. 194790/1982), the so-called runaway plasmid the copy number of which rapidly increases at elevated temperatures (cf. B. E. Uhlin et al., Gene, 6, 91, 1979 and Jpanese national publication of patent application under PCT No. 50057/1980), and a plasmid modified in the SD sequence or in the distance between the SD sequence and the translation startpoint to thereby increase the translation efficiency (Japanese Patent Application Kokai Tokkyo Koho No. 50893/1980, Japanese Patent Application No. 132524/1983).

The present inventors paid their attention to the above points and succeeded to modify in various ways the vector pGIF54 for exogeneous gene expression that had already been constructed (essentially the same plasmid as pGIF4 disclosed in Japanese Patent Application No. 86180/1982; an Escherichia coli strain transformed with said plasmid has been named SBMG105 and deposited with the Fermentation Research Institute, Agency of

Industrial Science and Technology, Ministry of International Trade and Industry, Japan, under the deposit numbers FERM P-6522 and FERM BP-282) in an attempt to construct <u>Escherichia coli</u> plasmid vectors

5 capable of realizing high-yield and high-efficiency production of useful polypeptides.

The details are described in the following.

As already mentioned, the SD sequence or the distance between the SD sequence and the translation

10 start codon (ATG) has an influence on the efficiency of heterologous polypeptides production. Therefore, the present inventors first attempted to modify the distance between the SD sequence and translation start codon ATG residing on the plasmid pGIF54. The plasmid

15 pGIF54 is a human gamma-interferon (hereinafter, hIFN-γ) expression vector containing a DNA fragment which comprises a gene (cf. Fig. 7) chemically synthesized and coding for a polypeptide having the amino acid sequence of hIFN-γ as connected downstream

20 from the β-galactosidase gene-derived <u>lac</u> UV5 promoter (cf., as to said plasmid, Japanese Patent Application No. 86180/1982). In said plasmid, the distance between the SD sequence and the translation start codon (ATG) for hIFN-γ is longer by 3 base pairs as compared with

25 the original <u>lac</u> promotor and β-galactosidase case. In view of the secondary structure of mRNA, the present inventors expected that modification of the above-

mentioned distance might lead to increase in the
efficiency of translation of the hIFN-γ polypeptide.
Therefore, they constructed a plasmid, pGIF54A, with
said distance increased by one base and a plasmid,

5   pGIF54C, with said distance reduced by one base and
studied the production of hIFN-γ in Escherichia coli
strains transformed with the respective plasmids. The
modification of the distance between the SD sequence
and ATG was accomplished by replacing the EcoRI-AvaII

10 fragment of pGIF54 with either of two chemically
synthesized DNA fragments (A) and (C) differing in
length, as illustrated in Fig. 1. The 5' end of the
upper strand of each of the chemically synthesized
double-stranded DNA fragments consists of AATT and

15 constitutes an EcoRI cohesive end. However, since the
base subsequent to AATT is A in the DNA fragment (C),
the plasmid pGIF54C with said fragment inserted therein
no more has the EcoRI site. (AATTC would constitute an
·EcoRI recognition site.)

20   In Escherichia coli strains transformed with these
plasmids, the hIFN-γ activity was determined and found
to be about 3 times and about 8 times with pGIF54A
and pGIF54C, respectively, as compared with the case of
pGIF54. It was thus found that the modification of the

25 distance between the SD sequence and ATG greatly
influences the productivity of the heterologous peptide
(hIFN-γ).

For further increase in productivity, the present
inventors then modified the plasmid pGIF54C by
modification of the promoter (introduction of the tac
promoter), introduction of the lac promoter repressor
gene i^q (cf. The Operon, pp. 31-88, editors: J. H.
Miller and W. S. Reznikoff, Cold Spring Harbor
Laboratory, 1978) and introduction of the runaway
plasmid (B. E. Uhlin et al., Gene, 6, 91, 1979) DNA.

The tac promoter is a hybrid promoter having the
-35 region for the Escherichia coli trp promoter and
the Pribnow box and the subsequent region of the
Escherichia coli lac promoter (cf. H. A. de Boer et
al., Proc. Natl. Acad. Sci. USA, 80, 21, 1983).
Characteristic features of this hybrid promoter are
that it has very strong promoter activity and that the
expression of structural genes which are downstream
therefrom is controlled by the lac promoter and repressor. The
latter means that the addition of an inducer such as
IPTG (isopropyl-β-D-thiogalactoside) can activate this
promoter. Whereas the construction of the tac promoter
has been described in the reference cited above, the
present inventors independently constructed the tac
promoter and further constructed a plasmid, ptacGIF54C,
which is capable of high production of useful
polypeptides under the control of said promoter.

Since it does not contain the lac promoter
repressor gene laci^q, the plasmid ptacGIF54C can

cause constitutional expression of an exogenerous gene (the hIFN-γ gene in the example to be described later) in the absence of an inducer such as IPTG. Furthermore, since the tac promoter in ptacGIF54C is

5   directed to the β-lactamase (Ap^r) gene, there is the possibility of the β-lactamase protein being produced in excess as well. Therefore, as will be mentioned in detail in the example, the laci^q gene was introduced into the plasmid ptacGIF54C, and a plasmid,

10   ptacGIF54Ci^q, with the directionality of the tac promoter reversed therein was constructed. An Escherichia coli transformant carrying this plasmid had a hIFN-γ (antiviral) activity about 16 times higher as compared with an Escherichia coli transformant carrying

15   pGIF54C (cf. Table 2).

Furthermore, the present inventors made an attempt to accomplish high production of useful polypeptides by introducing the runaway plasmid DNA said to cause rapid increase in plasmid copy number at elevated

20   temperatures. The runaways plasmid used was a gift from Dr. M. Inoue at New York State University. A host Escherichia coli strain carrying said plasmid has been named W620recA/pYM307 and deposited with the Fermentation Research Institute, Agency of Industrial

25   Science and Technology, Japan, under the deposit number FERM BP-345. pYM307 is derived from the runaway plasmid described by B. E. Uhlin et al. in Gene, 6, 91,

1979. This runaway plasmid of Uhlin et al. is now in wide use.

A host Escherichia coli strain (WA802/pY tacGIF) transformed with the plasmid pY-tacGIF constructed by introduction of a runaway plasmid DNA fragmnet was found to have an antiviral (hIFN-γ) activity about 16 times still higher as compared with an Escherichia coli transformant carrying ptacGIF54Ci^q, as shown later in the example.

In this way, the present inventors, as a result of intensive study, succeeded in constructing high expression vectors more suited for efficient production of useful polypeptides than expected.

The present invention, which has been completed on the basis of the above findings, provides an Escherichia coli plasmid which has, between the Shine-Dalgarno sequence and the translation start codon, a base sequence different from the corresponding, originally occurring region in the β-galactosidase oepron and contains, upstream therefrom, the region of a hybrid promoter between the Escherichia coli trp promoter and the lac promoter and, at an entirely independent site, a lac promoter repressor gene; a host procaryotic cell transformed with a plasmid containing a gene coding for the amino acid sequence of a useful polypeptide as inserted therein so that said gene can be expressed under the

control of the above hybrid promoter; and a culture
containing a polypeptide having the amino acid sequence
of human immune interferon as obtained by cultivating
a transformant selected from the group consisting of
5   WA802/ptacGIF54Ci^q and WA802/pYtacGIF.

In the accompanying drawings, Fig. 1 schematically
illustrates the process for the construction of the
plasmids pGIF54A and pGIF54C;

Fig. 2 schematically illustrates the process for
10 the construction of the plasmid ptacGIF54C;

Fig. 3 schematically illustrates the process for
the construction of the plasmid ptacGIF54Ci^q;

Fig. 4 is a schematic representation of the
restriction enzyme map of the plasmid pINIII containing
15 the _lac_ promoter repressor gene i^q;

Fig. 5 schematically illustrates the process for
the construction of the plasmid pY-tacGIF;

Fig. 6 is a schematic representation of the
restriction enzyme map of the runaway plasmid; and

20 Fig. 7 shows the amino acid sequence of hIFN-γ and
the base sequence of a chemically synthesized,
hIFN-γ-encoding gene.

The following examples illustrate the invention.
For the sake of brevity, the utility of the plasmids
25 according to the invention is shown by giving an
example of hIFN-γ polypeptide production. It is
obvious that the plasmids are also applicable to the
production of other useful polypeptides.

Examples

1. Construction of the plasmids pGIF54A and pGIF54C
   (cf. Fig. 1) and antiviral activity

   A. Construction of pGIF54A and pGIF54C

   The plasmid pGJF54 was once used for transforming
   the deoxycytosine-methylation deficient (dcm⁻)
   Escherichia coli strain GM271 (provided by the E. coli
   Genetic Stock Center at the Department of Human
   Genetics, Yale University School of Medicine, New
   Haven, Connecticut, USA; CGSC strain No. 6477). A
   5 μg portion of the pGIF54 plasmid DNA recovered from
   the transformant was dissolved in 50 μl of SalI
   solution (10 mM Tris hydrochloride buffer, pH 7.4,
   containing 10 mM MgCl$_2$, 150 mM NaCl and 10 mM
   mercaptoethanol). Following addition of 20 units of
   the restriction enzyme SalI and 10 units of PstI,
   digestion was effected at 37°C for 1 hour. The
   reaction mixture was subjected to agarose gel
   electrophoresis, and a PstI-SalI fragment comprising
   about 1300 base pairs was electrophoretically
   separated, treated with phenol and then precipitated by
   addition of alcohol. The precipitated DNA was dissolved
   in 50 μl of 6 mM Tris hydrochloride buffer (pH 8.0)
   containing 60 mM sodium chloride and 10 mM magnesium
   chloride, followed by addition of 10 units of AvaII.
   After digestion at 37°C for 1 hour, the reaction
   mixture was subjected to agarose gel electrophoresis.

The desired 436 base pair AVAII-SalI fragment was extracted electrophoretically, treated with phenol and precipitated by addition of alcohol.

Separately, 2 μg of pGIF54 DNA was dissolved in 50 μl of SalI solution and, following addition of 6 units of EcoRI and 10 units of SalI, digestion was effected at 37°C for 1 hour. The reaction mixture was subjected to gel agarose electrophoresis and the desired EcoRI-SalI fragment comprising about 3800 base pairs was extracted electrophoretically, treated with phenol and precipitated by addition of alcohol.

Furthermore, two DNA fragments, (A) and (C), having the respective base sequences shown in Fig. 1 and EcoRI and AvaII cohesive ends were synthesized by the solid method disclosed in Japanese Patent Application No. 86180/1982. A 20 pmol portion of each oligonucleotide was dissolved in 10 μl of 66 mM Tris hydrochloride buffer (pH 7.6) containing 6.6 mM magnesium chloride. Following addition of 1 μl of 4 mM ATP and 2 units of polynucleotide kinase, the phosphorylation of 5' OH was allowed to proceed at 37°C for 30 minutes.

Then, the above 3 DNA fragmens were subjected to ligation. Thus, the 436 base pair AvaII-SalI fragment and about 3800 base pair EcoRI-SalI fragment prepared in the above manner were dissolved in 10 μl of ligation solution (20 mM Tris hydrochloride buffer, pH 7.6, 10

mM MgCl$_2$). For constructing pGIF54A, 10 μl of the solution of a synthetic fragment (A) with the 5' OH phosphorylated or, for constructing pGIF54C, 10 μl of a solution of the synthetic fragment (C) with the 5' OH

5 phosphorylated was admixed with the above solution. Following addition of 1 unit of T4 DNA ligase, the ligation reaction was carried out at 16°C overnight. The DNA solutions thus obtained were used for transformation of _Escherichia_ _coli_ WA802 by the

10 conventional method. Transformant selection was conducted using a nutrient agar medium containing 40 μg/ml of ampicillin, and a number of ampicillin resistant transformants (WA802/pGIF54A and WA802/pGIF54C) were obtained. Isolation of the plasmid

15 DNA and analysis with restriction enzymes confirmed that these transformants carried the contemplated plasmid pGIF54A or pGIF54C.

B. Assay of the transformants for antiviral activity

     To quantify the expression of hIFN-γ, the parent

20 host strain WA802/pGIF54 and the above transformants WA802/pGIF54A and WA802/pGIF54C each was cultivated at 37°C overnight in 2 ml of L-broth (containing 40 μg/ml of ampicillin). A 0.2-ml portion of each culture thus prepared was inoculated into 5 ml of the same medium as

25 above. After incubation at 37°C until an OD$_{660}$ value of about 0.7, 0.05 ml of 100 mM IPTG was added and incubation was continued at 37°C for 2 hours. The

culture (1 ml) was centrifuged at 10,000 rpm for 5
minutes and the cells obtained were subjected to lysis
by adding 500 µl of 0.15 M phosphate buffer (pH 7.2)
containing 1 mg/ml of lysozyme and 50 mM sodium

5 chloride and allowing to stand in an ice bath for 10
minutes. The cells were then disrupted by 3
repetitions of rapid freezing in dry ice-ethanol and
rapid thawing in a constant-temperature vessel
maintained at 37°C. After centrifugation at 10,000 rpm

10 for 10 minutes, the supernatant was assayed for
antiviral activity by the method disclosed in Japanese
Patent Application No. 86180/1982. The results thus
obtained are shown in Table 1.

Table 1

15 Antiviral activity of each transformant

Transformant	Antiviral activity (IU/ml)	Ratio
WA802/pGIF54	13.7	1.0
WA802/pGIF54A	48.2	3.5
WA802/pGIF54C	108.2	8.0

As shown in Table 1, WA802/pGIF54A and
WA802/pGIF54C showed antiviral activity 3.5 times and
8.0 times stronger as compared with the original
transformant WA802/pGIF54. It was thus noted that the

25 base sequence between the SD sequence and the
translation start codon has a great influence on the

expression of heterologous polypeptides (in this example, hIFN-γ). The distance between the SD sequence and the translation start codon is longer by one base pair (T-A) in pGIF54A and shorter by one base pair

5 (C-G) in pGIF54C as compared with the plasmid before modification, namely pGIF54.

2. Modification of the promoter region: construction of the plasmid ptacGIF54C (cf. Fig. 2)

Fig. 2 is a schematic representation of the

10 construction of the plasmid ptacGIF54C by modifying the promoter region on the plasmid pGIF54C.

In Fig. 2, the plasmid pBR322-trp is a plasmid reconstructed, in the following manner, from the plasmid pSC101-trp obtained by the method described in

15 Gene, 1, 141-152, 1977. Thus, the pSC101-trp DNA was cleaved with HindIII, and a 4.5 Kb DNA fragment containing the Escherichia coli tryptophan promoter (trp promoter) was separated and partially digested with HpaI and an about 2.8 Kb DNA fragment was thereby

20 isolated. This DNA fragment was ligated with the longer DNA fragment obtained by digestion of pBR322 with HindIII-PvuII, whereby pBR322-trp was constructed. A pBR322-trp-carrying Escherichia coli transformant has been deposited with the Institute for Fermentation,

25 Osaka under the deposit number IFO-14281 and is readily available.

In the _trp_ promoter, a _TaqI_ site occurs between the -35 region and the Pribnow box. In the _lac_ promoter, a _HpaII_ site occurs at the same place. The protruding terminus remaining after cleavage with these two restriction enzymes is the same as $^{5'}CG^{3'}$. This facilitates ligation. By utilizing these cleavage sites, that portion of the _trp_ promoter which is upstream from the _TaqI_ site (containing the -35 region) and that portion of the _lac_ promoter which is downstream from the _HpaII_ site (containing the Pribnow box and the subsequent operator region) were ligated together. The details are described below.

A. Construction of the plasmid $ptrp^{-35}$

The plasmid pBR322-trp (2 µg) containing the structure covering the _trp_ promoter, _trp_ leader, _trpE_ and part of _trpD_ was dissolved in 50 µl of 33 mM Tris acetate buffer (pH 7.6) containing 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM dithiothreitol (hereinafter, TA solution). Following addition of 2 units of _TaqI_, digestion was effected at 65°C for 5 minutes. Under these conditions, the cleavage with _TaqI_ is incomplete, hence there occurred partial digestion. The desired DNA fragment is the about 3000 base pair DNA fragment resulting from cleavage of the _trp_ promoter at the _TaqI_ site between the -35 region and the Pribnow box and of pBR322 at the _ClaI_ site followed by joining. Such manner of joining

allows the $\underline{ClaI}$ site to remain, which is convenient for the subsequent ligation. For isolating this DNA fragment, the partially $\underline{TaqI}$-digested DNA was subjected to agarose gel electrophoresis, an about 3000 base pair DNA fraction was extracted electrophoretically, treated with phenol and precipitated by addition of ethanol. The precipitate thus obtained was dried and dissolved in 20 µl of 66 mM Tris hydrochloride buffer, pH 7.6, containing 6.6 mM magnesium chloride, 10 mM dithiothreitol and 0.4 mM adenosine triphosphate (hereinafter, ligation solution). Using 1 unit of T4 DNA ligase, ligation was effected at 16°C overnight. To the reaction mixture was added 2.5 volumes of ethanol, whereby a DNA precipitate was obtained. This was used for transforming $\underline{Escherichia}$ $\underline{coli}$ WA802. Transformant selection was conducted on a nutrient agar medium containing 40 µg/ml of ampicillin. Plasmid DNA separation of a number of ampicillin resistant transformants followed by plasmid checking using restriction enzymes gave the desired plasmid ptrp^{-35}.

B. Construction of ptacGIF54C

In the above-constructed ptrp^{-35}, the base just prior to the $\underline{TaqI}$ site downstream from the -35 region of the $\underline{trp}$ promoter is A and there occurs $\underline{ATCGA}$. This is in connection with the $\underline{ClaI}$ site ($\underline{ATCGAT}$) of pBR322. Therefore, there remains the $\underline{ClaI}$ site. By utilizing this $\underline{ClaI}$ site, ptrp^{-35} can be joined to the $\underline{HpaII}$

site of the <u>lac</u> promoter. This ptrp^{-35} has two <u>ClaI</u>
sites, so that partial <u>ClaI</u> digestion is required.
Accordingly, 2 µg of the ptrp^{-35} DNA was dissolved in
50 µl of TA solution, 6 units of <u>FstI</u> was added and

5   digestion was effected at 37°C for 10 minutes. The
enzyme was then inactivated by heating the reaction
mixture at 65°C for 15 minutes, and the mixture was
subjected to agarose gel electrophoresis. The desired
about 2300 base pair DNA fragment was extracted

10 electrophoretically, treated with phenol and
precipitated by addition of ethanol.

In the next place, for use in joining the
structure from the Pribnow box and thereon of the <u>lac</u>
promoter to the above ptrp^{-35}, 5 µg of the pGIF54C

15 plasmid DNA capable of hIFN-γ expression under the
control of the <u>lac</u> promoter was dissolved in TA
solution, <u>HindIII</u> and <u>HpaII</u> (10 units each) were added,
and digestion was effected at 37°C for 1 hour. The
subsequent agarose gel electrophoresis separated the

20 desired DNA fragment covering 352 base pairs from the
<u>HpaII</u> site within the <u>lac</u> promoter to the <u>HindIII</u> site
within the hIFN-γ gene. This fragment was extracted
electrophoretically, treated with phenol and
precipitated by addition of ethanol.

25 Then, to separate that structural gene portion
subsequent to the <u>HindIII</u> site of the hIFN-γ gene, 5 µg
of the same plasmid as above, i.e. pGIF54C, was

dissolved in 50 μl of 10 mM Tris hydrochloride buffer,
pH 7.4, containing 10 mM magnesium chloride, 150 mM
sodium chloride and 10 mM 2-mercaptoethanol
(hereinafter, SalI solution), 10 units of HindIII and

5 10 units of SalI were added, and digestion was effected
at 37°C for 1 hour. The reaction mixture was
subjected to agarose gel electrophoresis, and the
desired 155 base pair DNA fragment was extracted
electrophoretically, treated with phenol and

10 precipitated by addition of ethanol. Furthermore, 2 μg
of the pBR322 DNA was dissolved in 50 μl of SalI
solution, 6 units of PstI and 6 units of SlaI were
added, digestion was effected at 37°C for 1 hour, and
the reaction mixture was subjected to agarose gel

15 electrophoresis. The desired 1404 base pair DNA
fragment thus separated was extracted electro-
phoretically, treated with phenol and precipitated by
addition of ethanol. The four DNA fragments thus
obtained, namely the ClaI-PstI DNA fragment (about 2300

20 base pairs) containing the -35 region of the trp
promoter, the HpaII-HindIII DNA fragment covering 352
base pairs from the Pribnow box of the lac promoter and
thereon to about two thirds of the hIFN-γ gene, the 155
base pair HindIII-SalI DNA fragment covering the

25 remaining one third of the hIFN-γ gene and the 1404
base pair PstI-SalI DNA fragment, were dissolved in 20
μl of ligation solution. Using 1 unit of T4 DNA

ligase, ligation was effected at 16°C overnight.
Ethanol was added to the reaction mixture, and the
precipitate DNA thus obtained was used for transforming
Escherichia _coli_ WA802. The transformant

5   (WA802/ptacGIF54C) was selected on a nutrient agar
medium containing 40 μg/ml of ampicillin, whereby a
number of ampicillin transformants were isolated. The
plasmid DNA was separated from these transformants and
analyzed with restriction enzymes. It was thus

10   confirmed that said transformants carry the
contemplated plasmid ptaGIF54C. In the above analysis,
the contemplated plasmid was obtained in all the
isolates (18 strains), namely almost completely.

3.   Construction of the plasmid ptacGIF54Ci^q (cf.

15   Fig. 3)

ptacGIF54Ci^q is the plasmid constructed by
introduction of the _lac_ promoter repressor gene _laci_^{q}
into the plasmid ptacGIF54C obtained in Example 2-B and
reversing the _tac_ promoter region in said plasmid. Its

20   construction is schematically illustrated in Fig. 3.

The ptacGIF54C DNA (5 μg) was dissolved in 50 μl
of _Sal_I solution, followed by addition of 12 units of
_Cla_I and 12 units of _Sal_I. After digestion at 37°C for
1 hour, the reaction mixture was subjected to agarose

25   gel electrophoresis. The desired _Cla_I-_Sal_I DNA fragment
covering about 1000 base pairs and containing the _tac_
promoter and the γ-interferon gene as thus separated

was extracted electrophoretically, treated with phenol and precipitated with ethanol.

The introduction of the $\underline{laci}^q$ gene was then conducted. The $\underline{laci}^q$ gene-containing plasmid pINIII used in the practice of the invention, which was a gift from Dr. M. Inoue at New York State University, has the restriction enzyme map as shown in Fig. 4. An $\underline{Escherichia}$ $\underline{coli}$ transformant carrying said plasmid, JA221/pINIIIA1, has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the deposit number FERM BP-344.

The above pINIII plasmid DNA (2 µg) was dissolved in 50 µl of $\underline{Sal}$I solution, and 10 units of $\underline{Pst}$I and 10 unit of $\underline{Sal}$I were added. After digestion at 37°C for 1 hour, the reaction mixture was subjected to agarose gel electrophoresis, whereby a $\underline{Pst}$I-$\underline{Sal}$I DNA fragment covering about 5500 base pairs and containing the $\underline{laci}^q$ gene was separated. Said DNA fragment was extracted electrophoretically, treated with phenol and precipitated by addition of ethanol. Furthermore, 2 µg of the pBR322 DNA was dissolved in 50 µl of TA solution, 6 units of $\underline{Pst}$I and 6 units of $\underline{Cla}$I were added and, following digestion at 37°C for 1 hour, the mixture was subjected to agarose gel electrophoresis, whereby the desired 777 base pair $\underline{Pst}$I-$\underline{Cla}$I DNA fragment was separated. Said DNA fragment was

extracted electrophoretically, treated with phenol and precipitated by addition of ethanol.

The three DNA fragments obtained above (ClaI-SalI and PstI-SalI fragments from ptacGIF54C and PstI-SalI fragment from pBR322) were dissolved in 20 μl of ligation solution and, using 1 unit of T4 DNA ligase, ligation was effected at 16°C overnight. Addition of ethanol to the reaction mixture gave a DNA precipitate. Using this DNA, Escherichia coli WA802 was transformed. Selection of the transformant (WA802/ptacGIF54Ci^q) was conducted on a nutrient agar medium containing 40 μg/ml of ampicillin. A number of ampicillin resistant transformants were obtained. Plasmid DNA separation and analysis using restriction enzymes confirmed that 2 out of 5 such transformants carried the contemplated plasmid ptacGIF54Ci^q.

4. Introduction of the runaway plasmid DNA:

    construction of the plasmid pY-tacGIF (cf. Fig. 5)

The introduction of the runaway plasmid DNA into the plasmid ptacGIF54Ci^q is schematicaly illustrated in Fig. 5. The Escherichia coli strain pYM307 carrying the runaway plasmid (cf. Fig. 6) was a gift from Dr. M. Inoue at New York State University.

The ptacGIF54Ci^q DNA (2 μg) and 2 μg of the pYM307 runaway plasmid DNA were each dissolved in 50 μl of TA solution, and 12 units of EcoRI was added. After 1 hour of reaction at 37°C, the enzyme was inactivated

by heating at 65°C for 15 minutes. Addition of ethanol gave a DNA precipitate. After drying, each DNA precipitate was dissolved in 10 μl of ligation solution. The two solutions were mixed to make 20 μl and,

5 following addition of 1 unit of T4 DNA ligase, ligation was effected at 16°C overnight. Addition of ethanol to the reaction mixture gave a DNA precipitate, which was used for transforming Escherichia coli WA802. Since the pYM307 runaway plasmid has the kanamycin resistance

10 gene and ptacGIF54Ci^q has the ampicillin resistance, gene, the transformant (WA802/pY-tacGIF) carrying the contemplated plasmid composed of both the above plasmids joined together must exhibit both kanamycin resistance and ampicillin resistance. Therefore,

15 transformant selection was carried out on a nutrient agar medium containing 40 μg/ml of ampicillin and 20 μg/ml of kanamycin. Since the runaway plasmid is temperature-sensitive, the transformation was conducted at 28°C. From a number of transformants thus obtained,

20 plasmids were isolated and analyzed using restriction enzymes, and it was confirmed that 3 out of 6 such transformants carried the contemplated plasmid pY-tacGIF.

5. Assay of transformants for antiviral activity

25 To quantify the hIFN-γ expression in the transformants WA802/pY-tacGIF, WA802/ptacGIF54Ci^q and WA802/pGIF54C, each transformant (one strain selected

from each group of transformants carrying each
contemplated plasmid) was grown in 2 ml of L-broth
containing 40 μg/ml of ampicillin at 28°C overnight,
and 0.2 ml of the culture was inoculated into 4 ml of

5 L-broth having the same composition as above. After
cultivation at 32°C until an OD_{660} of about 0.7, 0.2
ml of 10 mM IPTG was added and the incubation was
continued at 37°C for further 3 hours.

   Bacterial cells were collected by centrifuging 1

10 ml of the above culture broth at 10,000 rpm for 5
minutes, 500 μl of 0.15 M phosphate buffer (pH 7.2)
containing 1 mg/ml lysozyme and 50 mM sodium chloride
was added thereto and the mixture was allowed to stand
on an ice bath for 10 minutes to thereby cause lysis.

15 Thereafter, rapid freezing in dry ice-ethanol followed
by rapid thawing in a constant-temperature bath
maintained at 37°C was repeated three times to thereby
cause cell disruption, followed by centrifugation at
10,000 rpm for 10 minutes. The supernatant was assayed

20 for antiviral activity by the method disclosed in
Japanese Patent Application Kokai Tokkyo Koho No.
86180/1982. The results thus obtained are shown in
Table 2.

Table 2

Antiviral activity of each transformant

Transformant	Antiviral activity (IU/ml)	Ratio
WA802/pGIF54C	74	1.0
WA802/ptacGIF54Ci^q	1,170	15.8
WA802/pY-tacGIF	18,724	253.0

As shown in Table 2, the plasmid ptacGIF54Ci^q constructed by introduction of the new promoter (tac promoter) and the lac promoter repressor gene into the lac-dependent expression vector pGIF54C brought about an antiviral activity about 16 times higher as compared with pGIF54C, and the plasmid pY-tacGIF constructed by further introduction of the runaway plasmid DNA brought about an antiviral activity still 16 times higher as compared with ptacGIF54Ci^q (253 times higher as compared with the original pGIF54C). As compared with the starting plasmid pGIF54, the final plasmid pY-tacGIF is thus about 2,000 times higher in antiviral activity production capacity. This indicates that the improvement in the plasmid vector in accordance with the present invention are surprisingly effective in the production of heterologous polypeptides.

The antiviral activity shown in Table 2 disappeared upon treatment at pH 2 and upon neutralization with an hIFN-γ-specific antibody. Said activity,

therefore, undoubtedly reflects the hIFN-γ production.
Although there have been disclosed various methods of
interferon production using the rDNA technology, it is
for the first time that such novel vectors that enable
5   high production of useful polypeptides have actually
been constructed as a result of intensive plasmid
modification efforts used by the present inventors.

Claims:

1. An _Escherichia_ _coli_ plasmid which has, between the Shine-Dalgarno sequence and the translation initial codon, a base sequence different from the corresponding, originally occurring region in the β-galactosidase operon and contains, upstream therefrom, the region of a hybrid promoter between the _Escherichia_ _coli_ _trp_ promoter and the _lac_ promoter and, at an entirely independent site, a _lac_ promoter repressor gene.

2. A plasmid as claimed in claim 1, wherein a plasmid DNA capable of causing an increase in plasmid copy number (runaway plasmid DNA) is inserted therein.

3. A plasmid as claimed in claim 1 or 2, wherein a gene coding for the amino acid sequence of a useful polypeptide is inserted therein so that said gene can be expressed under the control of said hybrid promoter.

4. A plasmid as claimed in claim 3, wherein said useful polypeptide is human immune iterferon.

5. A plasmid as claimed in claim 4, wherein the gene coding for the amino acid sequence of human immune interferon is a chemically synthesized gene or a gene prepared from an mRNA isolated and purified from the human spleen tissue or peripherial blood lymphocytes.

6. A plasmid as claimed in claim 5, wherein the chemically synthesized gene has the base sequence given in Fig. 7.

7. The plasmid of any of claims 1 and 3-6, which is selected from the group consisting of ptacGIF54Ci^q plasmid species.

8. The plasmid of any of claims 2-6, which is selected from the group consisting of pY-tacGIF plasmid species.

9. A host procaryotic cell transformed with the plasmid of any of claims 3-8.

10. A transformant as claimed in claim 9, wherein the host procaryotic cell is of _Escherichia coli_.

11. A transformant as claimed in claim 9, which is selected from the group consisting of WA802/ptacGIF54Ci^G and WA802/pYtacGIF.

12. A culture containing a polypeptide having the amino acid sequence of human immune interferon as obtained by cultivating the transformant of claim 11.

FIG.1

Construction of pGIF54A & pGIF54C

(A)

5'AATTCTATGTGCTAGTGCCAG
       GATACACGGTCCTG

(C)

5'AATTATGTGCTACTGCCAG
      TACACGATGACGGTCCTG

chemically synthesized
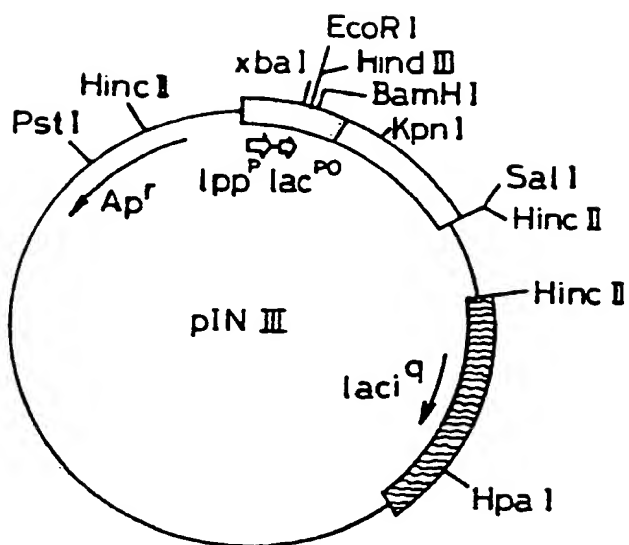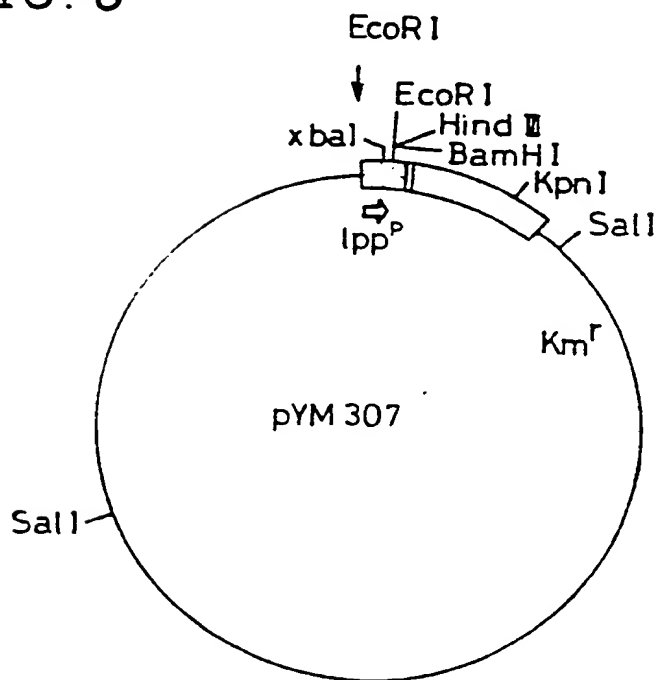DNA fragment
(A) or (C)
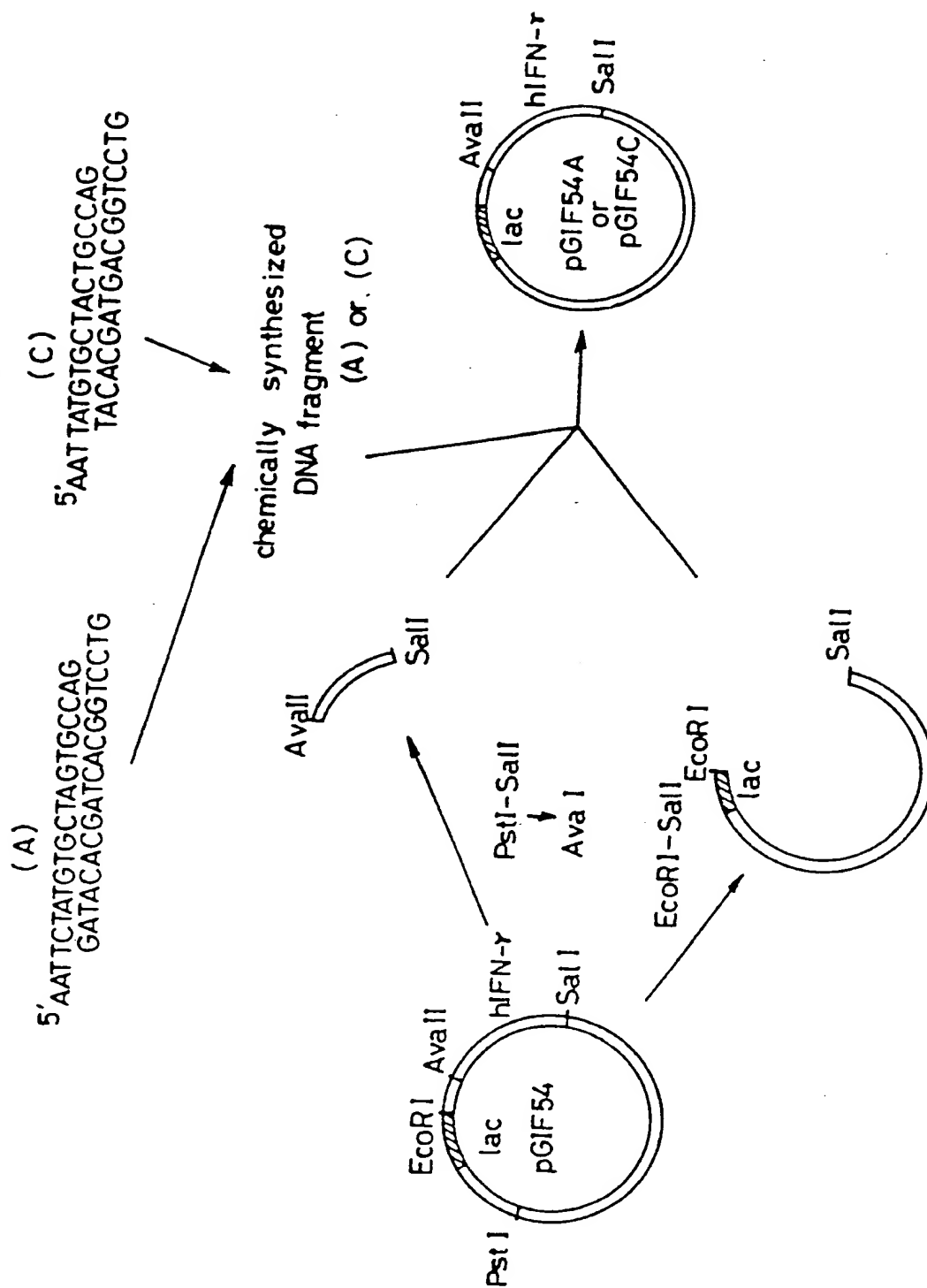
FIG.2
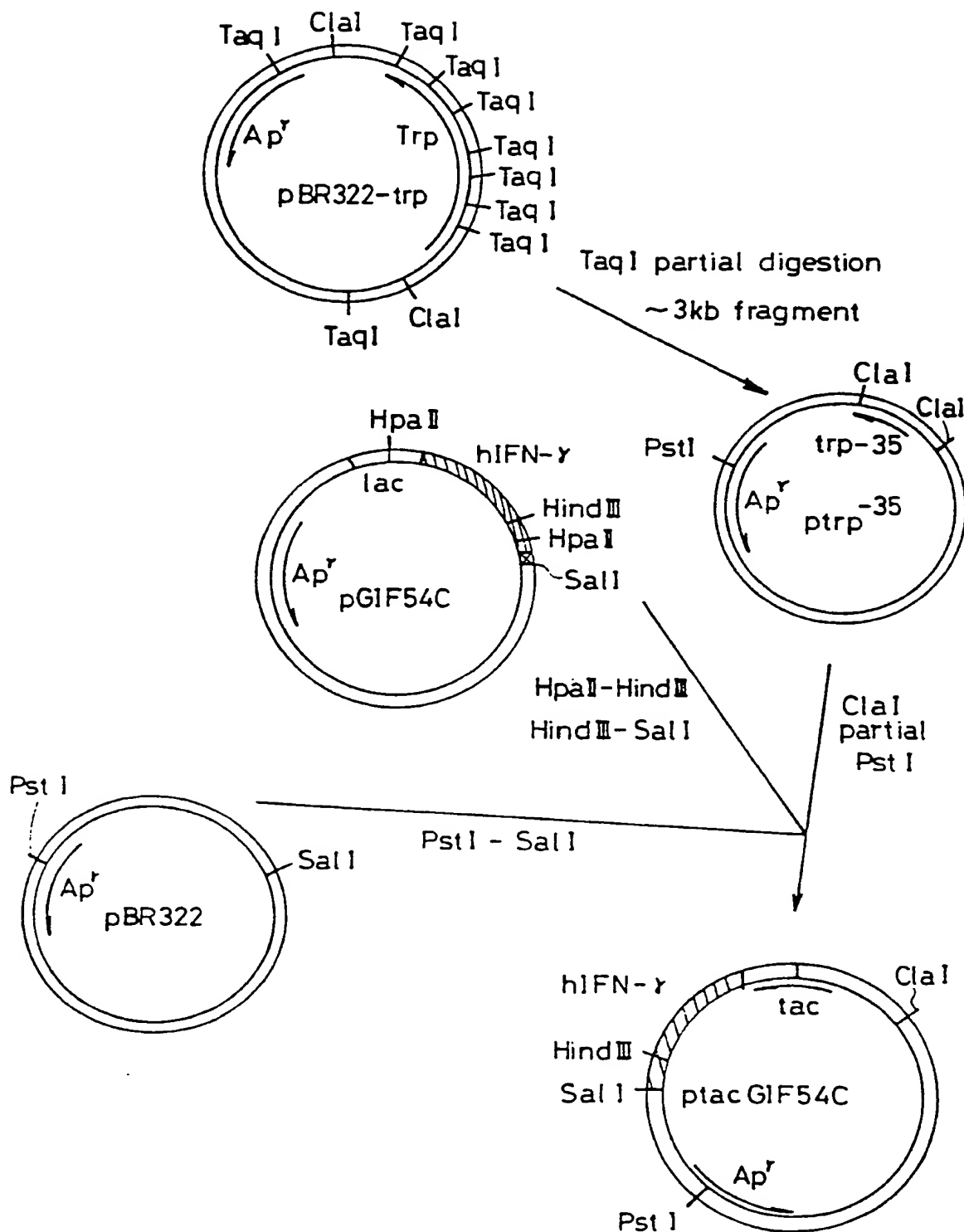
FIG. 3
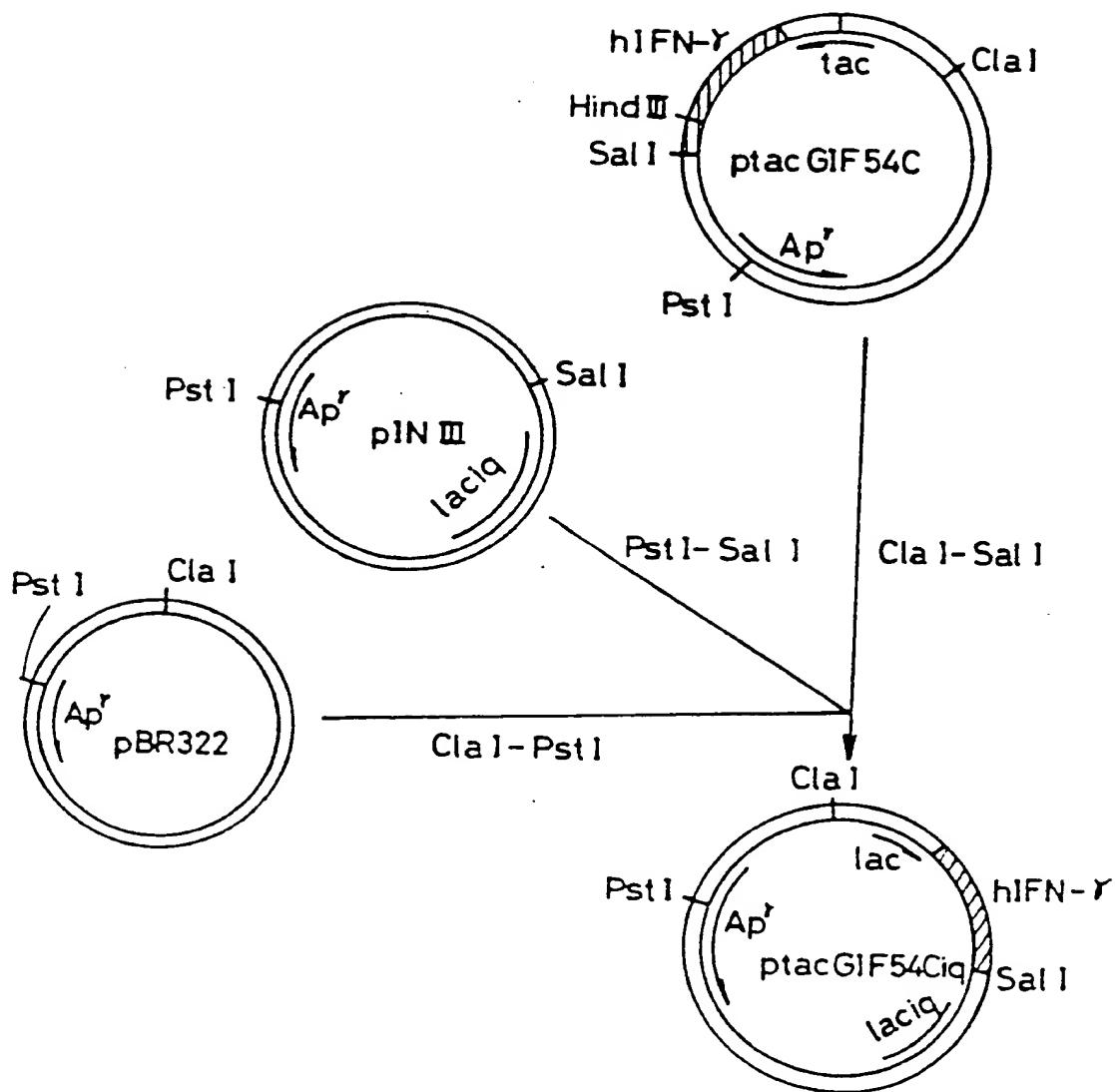
FIG. 4

FIG. 6

FIG.5

(Construction of pY tacGIF plasmid)
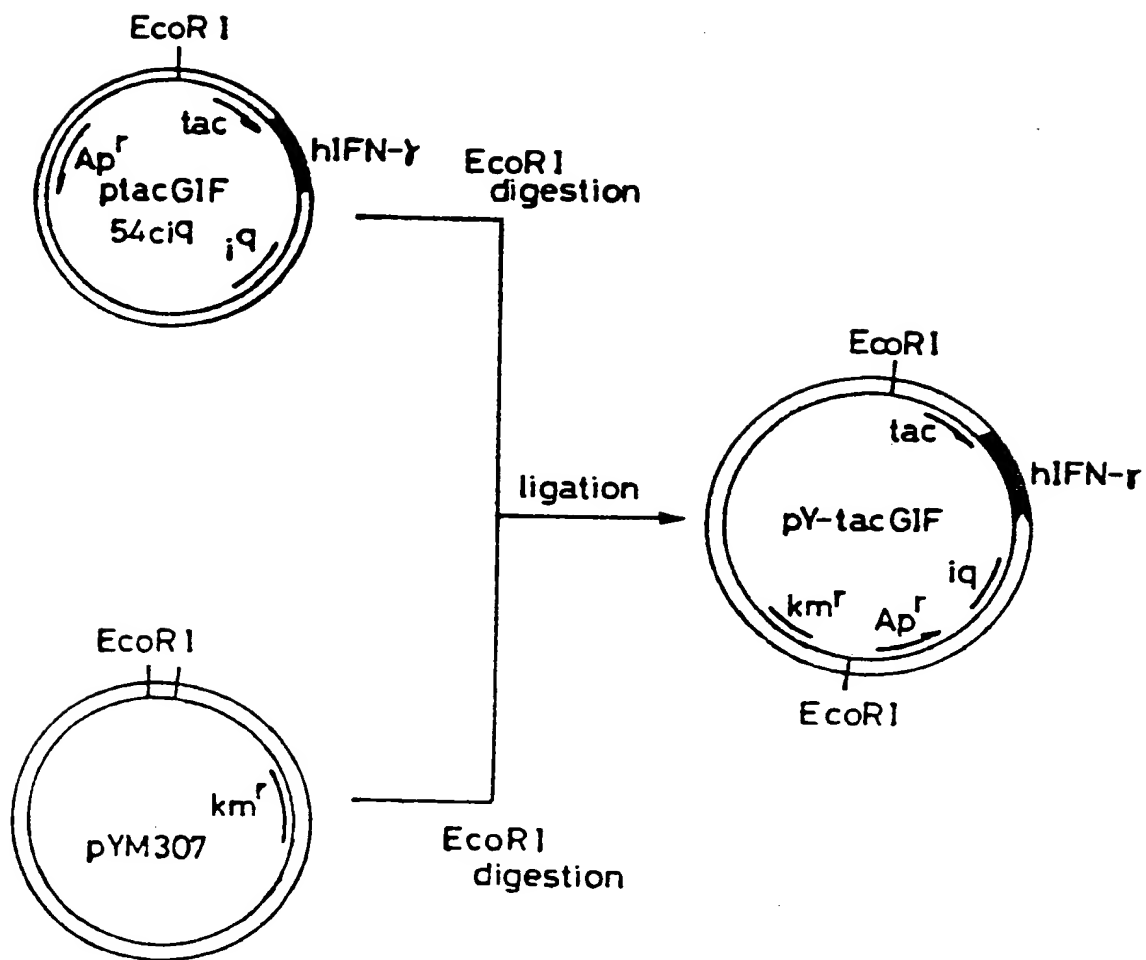
FIG.7

Met Cys Tyr Cys Gln Asp Pro Tyr Val Lys Glu Ala Glu Asn Leu Lys Lys Tyr Phe Asn Ala Gly His Ser Asp
1   10   20
5' AATTC ATG TGC TAC TGC CAG GAC CCA TAC GTG AAG GAA GCT GAA AAC CTG AAG AAA TAC TTC AAC GCT GGT CAT TCT GAC
3'   G TAC ACG ATG ACG GTC CTG GGT ATG CAC TTC CTT CGA CTT TTG GAC TTC TTT ATG AAG TTG CGA CCA GTA AGA CTG

Val Ala Asp Asn Gly Thr Leu Phe Leu Gly Ile Leu Lys Asn Trp Lys Glu Glu Ser Asp Arg Lys Ile Met Gln
                        30   40
GTT GCT GAC AAC GGT ACT CTG TTC CTG GGT ATC CTG AAA AAC TGG AAA GAA GAA TCT GAC CGT AAA ATC ATG CAG
CAA CGA CTG TTG CCA TGA GAC AAG GAC CCA TAG GAC TTT TTG ACC TTT CTT CTT AGA CTG GCA TTT TAG TAC GTC

Ser Gln Ile Val Ser Phe Tyr Fhe Lys Leu Phe Lys Asn Phe Lys Asp Asp Gln Ser Ile Glu Gln Lys Ser Val Glu
50   60   70
TCT CAG ATC GTT TCT TTC TAC TTC AAG CTG TTC AAA AAC TTC AAG GAC CAG TCT ATC CAG AAA TCT GT T GAA
AGA GTC TAG CAA AGA AAG ATG AAG TTC GAC AAG TTT TTG AAG TTC CTG GTC AGA TAG GTC TTT AGA CAA CTT

Thr Ile Lys Glu Asp Met Asn Val Lys Phe Phe Asn Ser Asn Lys Lys Lys Arg Asp Asp Phe Glu Lys Leu The
                        80   90
ACT ATC AAG GAA GAC ATG AAC GT T AAG TTC TTC AAC TCT AAC AAG AAG AAG CGT GAC GAC TTC GAA AAG CTT ACT
TGA TAG TTC CTT CTG TAC TTG CAA TTC AAG AAG TTG AGA TTG TTC TTT TTC GCA CTG CTG AAG CTT TTC GAA TGA

Asn Tyr Ser Val Thr Asp Leu Asn Val Gln Arg Lys Ala Ile His Glu Leu Ile Gln Val Met Ala Glu Leu Ser
100   110   120
AAC TAC TCT GT T ACT GAC CTT AAT GTA CAG CGT AAA GCT ATC CAT GAA CTG ATC CAG GTT ATG GCT GAA CTG TCC
TTG ATG AGA CAA TGA CTG GAA TTA CAT GTC GCA TTT CGA TAG GTA CTT GAC TAG CAA TAC CGA CTT GAC AGG

Pro Ala Ala Lys Thr Gly Lys Arg Lys Arg Ser Gln Met Leu Phe Ara Gly Arg Arg Ala Ser Gln Slp
                        130   140   146
CCG GCT GCT AAA ACT GGT AAA AGA AAG CGT AAA AGA TCT CAG ATG CTG TTC CGT GGT CGT TCT CAG TAA G 3'
GGC CGA CGA TTT TGA CCA TTA CTT TCT GCA TTT TCT AGA GTC TAC GAC AAG GCA CCA GCA GCA CGA AGA GTC ATT CAGCT 5'

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